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Phenylalanine requirement and blockage in its biosynthesis in C. Elegans

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PHENYLALANINE REQUIREMENT AND BLOCKAGE IN ITS BIOSYNTHESIS IN *C. ELEGANS*.

A Thesis

Presented to

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San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Sripriya Ravi

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Abstract

PHENYLALANINE REQUIREMENT AND BLOCKAGE IN ITS BIOSYNTHESIS IN *C. ELEGANS*

By Sripriya Ravi

Phenylalanine requirement, optimal phenylalanine-tyrosine combination and the sparing effect between the two amino acids was determined in the free-living nematode, *C. elegans*. The optimal requirement of phenylalanine ranged between 1.1-4.2 mg/ml. The optimal phenylalanine - tyrosine combination was established as 0.53: 0.27 mg/ml. Phenylalanine completely spared tyrosine requirement at the optimal range established. At 0.27 mg/ml tyrosine spared as high as 75 % of phenylalanine requirement. The site of blockage in the biosynthetic pathway of phenylalanine was determined by supplementing precursors of phenylalanine in the medium. 85 - 92 % blockage was noticed with phenylpyruvic acid. Major blockage (100 %) occurred with prephenic acid. Conclusive evidence of additional blockage with shikimic acid and chorismic acid was not indicated.

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PREFACE

The following is a publication style thesis. The second chapter is written in journal format according to the guidelines published by the journal, Nematology. Chapters 1 and 3 are written according to the guidelines outlined in the Publication Manual of the American Psychological Association (4th edition) 1994.

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Chapter 1

Review of Literature

Caenorhabditis elegans - An introduction

Caenorhabditis elegans (C. elegans) is a microscopic free-living bacteriovorous nematode or roundworm. Nematodes are smooth skinned, unsegmented worms with a long cylindrical body shape, which tapered at both ends (Blaxter, 2000). It belonged to the phylum Nematoda. This phylum is made up of roundworms and threadworms, which may be free-living or parasitic, aquatic or terrestrial. Within the phylum, *C. elegans* belonged to Rhabditida (an order of the most free-living soil nematodes) and family Rhabditidae (Fitch & Thomas, 1997). Recent analyses suggested that nematodes were the basal branch of a clade of molting animals, called the ecdysozoa, which also included tardigrades, onycophorans and arthropods (Aguinlado, Turbeville & Linford, 1997). The phylogenetics of Rhabditida and of Caenorhabditis is an active field of research (Burglin, Lobos & Blaxter, 1998).

C. elegans exists in two sexual forms- hermaphrodite and male. The hermaphrodites produce both sperms and eggs that are self-fertilizing. The adult hermaphrodite is about 1.2 mm in length and 0.1 mm in width (Marx, 1984). Since the nematode bodies are transparent, their development can be followed by differential interference contrast (DIC) microscopy. This has allowed the establishment of their complete cell lineage. The adult hermaphrodite has 959 somatic nuclei while the adult

male has 1031 somatic nuclei (Sulston, Schierenberg, White & Thomson, 1983). A large variety of different cell types could be seen in *C. elegans* - muscles, intestines, hypodermis, and neurons. It is also the only animal for which the entire wiring of its nervous system is known. In particular, 302 neurons have been described which can be grouped into 118 morphologically different types (White, Southgate, Thompson & Brenner, 1986).

C. elegans have a relative short generation time. The fertilized egg develops into a mature animal in only 3.5 days (Marx, 1984). After hatching, the development progresses through four larval stages (L1- L4), before it becomes an adult (Kenyon, 1988). The growth and reproduction of *C. elegans* is very sensitive to temperature. The optimal temperature is 18 ^{0}C to 22 ^{0}C (Croll & Mathews, 1977). Under ideal conditions, the total life span of this animal is between 20 - 30 days (Bolla, 1987). Under special environmental conditions (such as crowding or absence of food), an alternative developmental pathway, the dauer larval stage, can be chosen. In this stage the life span of *C. elegans* could be extended (Riddle, 1982).

C. elegans is a microscopic soil nematode. In its natural environment, it depends on yeast, fungi, algae and bacteria in the soil for food. In the laboratory, *C. elegans* can be easily cultivated in petri-plates with *Escherichia coli* as food (Edgar & Wood, 1977). Mass populations could be cultivated in an axenic (germ-free) liquid media (Dougherty, Hanson, Nicholas, Mollett & Yarwood, 1959). The stock culture could also be easily stored and frozen at - 70 ^{0}C or in liquid nitrogen (Burglin *et al.*, 1998).

The *C. elegans* Genome

C. elegans is the first animal to have its entire genome sequenced. The genome size of *C. elegans* is 100 megabases or 1/30th the size of the human genome. It has six chromosomes, five autosomes and a sex chromosome, all of similar size. About 1/2 of the genes have similarity to genes in other organisms and some are homologs of human disease genes (The *C. elegans* Sequencing Consortium, 1998;Wilson, 1998). A number of human disease gene homologs in *C. elegans* have already been studied to some extent. For example, sel-12 gene is a homolog of human presenilin genes 1 and 2. Mutations in these genes are responsible for early onset of the neuro-degenerative disorder Alzheimer's disease (Alzheimer's Disease Collaborative Group, 1995). Other examples include disease genes such as cystic fibrosis, amyotropic lateral sclerosis, dementia, pancreatic carcinoma, polycystic ovarian disease and many more (see review -Aboobaker & Blaxter, 2000).

Comparative Gene Identity (CGI) studies have investigated the similarity of the completed *C. elegans* genome to human genes. The *C. elegans* Sequencing Consortium (1998) had reported 36 % matches of *C. elegans* genes with human genes. When 1880 human proteins were compared with 20000 predicted nematode proteins, Wheelan, Boguski, Duret and Makalowski (1999) reported that 44 % of human orthologs had convincing nematode counterparts. The *C. elegans* proteome (protein sequences identified in the *C. elegans* genome project) was used as an alignment template to assist in novel human gene identification from human Express Sequence Tags (EST) nucleotide

databases. At least 83 % of *C. elegans* proteome was reported to potentially have human orthologs (Lai, Chou, Ch'ang, Liu & Lin, 2000).

C. elegans could be used to screen for the adverse effects of drugs on mutant genes. Increased sensitivity, resistance or behavioral changes in relation to drugs that affect the genetic or metabolic pathway in the nematode could thereby be identified (Miller, *et al.*, 1996). *C. elegans* could also be used as a model for studying nematode parasitic infections in humans as some of these nematodes species are closely related (Grant, 1992).

C. elegans as a Research Model in Nutrition

C. elegans is an ideal model for nutritional research for there are numerous similarities in its digestive system to humans. The mouth leads to the pharynx and the esophagus followed by the intestines, rectum and anus. Initial digestion is extra cellular, and intracellular digestion occurred in the intestines. Wastes from the gut diffuse across the intestinal wall and are gathered by special cells known as renette cells. The solidified wastes are passed through the rectum and anus (Bolla, 1987).

Over the years the nutritional requirements of *C. elegans* and *Caenorhabditis briggsae* (*C. briggsae*) have been extensively studied. Margaret Briggs Gochnauer isolated the free-living, self-fertilizing, hermaphroditic nematode, *Caenorhabditis briggsae*, from the Stanford soil in 1944. It was brought to the Berkeley area and maintained and studied intensively by Dougherty and co-workers (Dougherty *et al.*, 1959). A major objective of their work was to develop a chemically defined medium

capable of supporting the indefinite axenic culture of *C. briggsae* and related rhabditid nematodes. Axenic cultivation of *C. briggsae* was first established (Buecher, Hansen & Yarwood, 1966) with the development of *C. briggsae* Maintenance Medium (CbMM). The CbMM was an extremely rich medium being composed of 53 components, all present at high concentrations. However the CbMM by itself could not support the growth and reproduction of *C. briggsae*. The requirement of sterol (Lu *et al.*, 1977, Hieb & Rothstein, 1968), heme (Hieb, Stockstad & Rothstein, 1970) and potassium acetate (Lu *et al.*, 1978) was established. Hieb and Rothstein (1968) demonstrated that sterol was an essential nutrient for the nematode, which could be supplied by cholesterol, ergosterol or β-sitosterol. Cytochrome c, an iron containing porphyrin compound, was shown to be another essential nutrient for the nematodes (Hieb, Stockstad, & Rothstein, 1970). The highest population growth in the nematodes was determined when the medium was supplemented with 200 μg/ml of cytochrome c (Chang, Lu, Smith & Belo, 1988). Chang *et al.*, (1988) also demonstrated that a higher population growth could be attained with a larger surface area exposure (12 cm^2 diameter in culture tubes instead of 10 cm^2). Lipid related growth factors such as sodium oleate, sodium stearate, tween 80, ethanol, n-propanol or potassium acetate could also be used instead of potassium acetate as energy source for the nematode (Lu *et al.*, 1978).

The final axenic medium, the *C. elegans* Maintenance Medium (CeMM) (Appendix I) was developed by Lu & Goetsch (1993) with the establishment of glucose as an alternative energy source. The CeMM consisted of 56 components and is currently being used for the axenic culture of *C. elegans*. *C. elegans* and *C. briggsae* are closely

related species. Friedman, Platzer, & Eby (1977) used electrophoretic analysis in combination with morphological, genetic and nutritional studies to differentiate between the two species. They were able to demonstrate that *C. elegans* and *C. briggsae* differed only by genetic composition and the ability to interbreed. Nicholas (1984) reported that some earlier papers published on *C. briggsae* might in fact describe work on *C. elegans*.

Many studies have established the nutrient essentiality and quantified the nutrient requirement for the optimal growth of the nematodes in an axenic environment. Glucose and trehalose have been reported to be the most readily utilized carbohydrate forms in *C. briggsae* (Hansen & Buecher, 1970).

The requirement of vitamins such as riboflavin, thiamin, folic acid, nicotinamide, pantothenic acid and pyridoxine for optimal growth of the nematodes was determined by various studies. Augustin, Lu, Belo, Smith & Saltmarch (1994) demonstrated the optimal requirement of thiamin. They reported that maximum population growth in the nematodes was attained at 0.75 µg/ml of thiamin when glucose was used as an energy source. Whereas, when acetate was used, ten times more thiamin (7.5 µg/ml) was required for the optimal growth of the nematodes.

Folic acid deficiency led to the accumulation of formimino - L- glutamic acid in the nematode tissue (Lu, Hieb & Stockstad, 1974). Both folic acid and vitamin B_{12} were required for the conversion of homocysteine to methionine (Lu, Hieb & Stockstad, 1976). A decrease in the growth of the nematodes in a medium containing folic acid antagonist,

aminopterin, further concurred the essentiality of this vitamin (Vanfleteren & Avau, 1977).

Sun, Lu, Tseng and Smith in 1986 demonstrated that Vitamin B_6 was essential for the growth and reproduction of *C. elegans*. Vitamin B_6 in all the three forms (pyridoxamine, pyridoxine and pyridoxal phosphate) promoted optimal growth of *C. elegans* at a concentration of 1.5×10^{-2} nmol/ml of the medium. Tryptophan metabolites (xanthurenic acid and kynurenic acid) accumulated in the Vitamin B_6 deficient culture medium.

Niacin was another vitamin that was demonstrated to be required for the growth of nematodes by Li, Lu, McProud and Sucher (1995). Supplementation with nicotinamide, nicotinic acid or a combination revealed that nicotinic acid promoted growth better than nicotinamide (1.5 -190 µg/ml). Maximum growth in population was attained when both nicotinic acid and nicotinamide were supplemented at 1.5 µg/ml each. The tryptophan to niacin conversion was also determined in this study, which was 500:1. This value was quite low compared to humans, who have a ratio of 60:1.

In1997, Pothana, Lu and Gordon established the requirement for riboflavin (37.5 µg/ml). A higher population growth was demonstrated when the medium was supplemented with the coenzyme flavin mononucleotide.

C. elegans were also shown to require magnesium (73 µg/ml), sodium (300 µg/ml), potassium (530 µg/ml), manganese (6.3 µg/ml), calcium (1500 µg/ml), and

copper (7.2 μg/ml) (Lu, Cheng & Briggs, 1983). Weber and Lu (1992) further reported that the level of zinc needed to support optimal population growth ranged from 4.9 - 37 μg/ml.

Vanfleteren (1973) established that *C. briggsae* had the same essential and non-essential amino acids requirements as higher organisms such as rats and humans. The essential amino acids were demonstrated to be arginine, histidine, lysine, tryptophan, phenylalanine, methionine, threonine, leucine, isoleucine, and valine. Perelman and Lu (2000) established the quantitative requirements for all the three branched chain amino acids (leucine, isoleucine and valine) and studied the interactions between them. The optimal range of leucine, isoleucine and valine requirements were 0.72 - 2.8, 0.86 - 1.7, and 0.51 - 4.1 mg/ml respectively. Quantitative levels for each of the other essential amino acids has not yet been determined.

The optimal levels of amino acids used in the *C. elegans* maintenance (CeMM) medium is given in Table I.

Table I. **Concentrations of Amino acids in the *C. elegans* Maintenance Medium (Lu & Goetsch 1993)**

Essential Amino acids (mg/ml)		Non- Essential Amino acids (mg/ml)	
L-Arginine	0.98	L- Tyrosine	0.27
L- Histidine	0.28	L- Alanine	1.4
L- Lysine HCl	1.3	L- Aspartic acid	1.6
L- Tryptophan	0.18	L- Cysteine.HCl.H_2O	0.03
L- Methionine	0.39	L- Glutamate (Na). H_2O	0.55
L- Threonine	0.72	L- Glutamine	1.5
L- Leucine	1.4	L- Glycine	0.72
L- Isoleucine	0.86	L- Proline	0.65
L-Valine	1.0	L- Serine	0.79
L- Phenylalanine	0.80		

The Metabolic Role of Phenylalanine/Tyrosine

Phenylalanine is metabolized via three possible biochemical pathways in humans - incorporation in proteins, hydroxylation to tyrosine and transamination to phenylpyruvic acid (Nyhan, 1967). A block in any of the three pathways would lead to accumulation of phenylalanine in the blood. Inability to incorporate phenylalanine into protein owing to lack of activation or to a defect in transfer RNA would theoretically be incompatible with life. Inability to form tyrosine and accumulation of phenylalanine metabolites in toxic concentrations are characteristics of the disease, phenylketonuria

(Nyhan, 1967). A block in the transamination of phenylalanine to phenylpyruvic acid could also lead to accumulation of phenylalanine. This, however, does not normally happen, since greater phenylalanine concentrations were required to saturate the transaminase than the hydroxylase (Kaufman, 1959).

Tyrosine was metabolized by deamination to yield 4- hydroxy phenylpyruvate and then to homogentisate. A monooxygenase enzyme catalyzed this reaction. Oxidative fusion of the aromatic ring of homogentisate yields maleyl acetoacetate, which was isomerized to fumaryl acetoacetate. Fumarate and acetoacetate were the final end products after hydrolysis of fumaryl acetoacetate (Nyhan, 1967).

Tyrosine was a precursor for many neurogenic amines such as epinephrine and norepinephrine (Lehninger, Nelson & Cox, 1983). Phenylalanine/tyrosine were required for the synthesis thyroid hormones, thyroxine and tri-iodo thyronine, and the pigment melanin. Melanins are a family of high molecular weight polymers that contain various metabolites of dopaquinone. The hydroxyl group of tyrosine was an important catalytic site of many enzymes and also provided a site for reversible phosphorylation catalyzed by protein kinases (Bender, 1985).

Requirements of Phenylalanine/ Tyrosine

Phenylalanine was shown to be nutritionally essential for nematodes *Angiostrongylus costaricencis* (Hata, 1994), and *Neoaplectana glaseri* (Jackson, 1973), *Rhabditis maupasi* (Brockelman & Jackson, 1978) *Aphelenchoides rutgersi* (Myers & Balasubramaniam, 1973), and *Caenorhabditis briggsae* (Vanfleteren, 1973). Tyrosine

was not demonstrated to be an essential nutrient in any of these nematodes. Tyrosine was a marginally essential nutrient (as manifested by delayed reproduction and reduced population growth) for *Neoaplectana glaseri* but not for *Angiostrongylus costaricencis*. A medium deficient in phenylalanine suppressed pigment production but did not affect growth in bacteria such as *Legionella pneumophila* and was hence not regarded as essential for this species (Tesh & Miller, 1981). However, George, Pine, Reeves & Harell, (1980) demonstrated phenylalanine to be essential for this species as evidenced by adequate growth of the bacterium.

The requirements of phenylalanine/tyrosine for various organisms has been summarized in Table II.

Table II. **A Comparison of Phenylalanine/Tyrosine Requirements for Different Species**

Animals	Phenylalanine/tyrosine (g/kg diet)	Tyrosine
Vertebrates		
Humans (25-50 yr)[1#]	4.2	
Chick growth (0-6 weeks)[2#]	10	
Rat growth (weanling) [2]#	8.0	
Swine (105 Kg) [2]#	12	
Hamster (weanling) [2]#	8.3	
Invertebrates		
Post larval tiger shrimp[3]	0.0014	*
Bacteria		
Helicobacter pylori[4]	0.017 mg/ml	*
Nematodes		
Neoaplectana glaseri[5]	0.08 mg/ml	0.08mg/ml
Aphelenchoides rutgersi[6]	0.15 mg/ml	*
Rhabditis maupasi[7]	0.08 mg/ml	*
Caenorhabditis elegans [8]**	0.80 mg /ml	0.27 mg/ml

Values given as total aromatic amino acids

* Tyrosine was not nutritionally essential

[1] Basile-Filho, El-Khoury, Beaumier, Wang & Young (1997)

[2] Mclarney, Pellett and Young (1996)

[3] Millamena, Teruel, Kanazawa & Teshima, 1999

[4] Reynolds & Penn, 1994

[5] Jackson, 1973

[6] Myers & Balasubramaniam, 1971

[7] Brockelman & Jackson, 1978

[8] ** Lu & Goetsch, 1993 (based on CeMM values)

Sparing Effect between Phenylalanine and Tyrosine

Tyrosine could spare or substitute phenylalanine in some animals. A study conducted with kittens on the sparing effect of tyrosine on their phenylalanine requirement indicated that tyrosine was able to spare about 42 % of the phenylalanine requirement (Williams, Morris & Rogers, 1987). The NRC (1978) reported that tyrosine could replace phenylalanine up to 50 % in domestic animals. Sasse and Baker (1972) reported a 42.5 % sparing effect of tyrosine in chickens. The requirement of phenylalanine was reduced due to the sparing effect of tyrosine by 53 % on a weight basis in rainbow trout as well (Kim, 1993). In lactating sow, when the phenylalanine requirement was assessed in the presence of excess tyrosine, it was demonstrated that tyrosine could supply up to 48 % of its total aromatic amino acid requirement (Lellis & Speer, 1987).

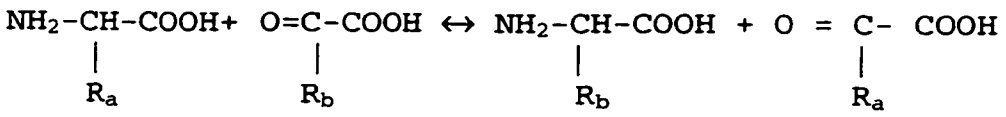
Biosynthesis of Amino acids

All amino acids were synthesized from the degradative products of glucose such as pyruvate and α-ketoglutarate in prokaryotes. The first step called amination, involved addition of an amino group to a keto acid. For example,

$$\text{Pyruvate} + NH_3 \rightarrow \text{Alanine}$$

$$\underline{\text{Or}}\ \alpha\text{-Ketoglutarate} + NH_3 \rightarrow \text{Glutamate}$$

An amino group may also be added to a keto acid by transfer from an amino acid, by a process termed transamination. For example,



By either amination or transamination, all 20 different amino acids could be synthesized from degradation products of glucose and be then available for assembly into proteins (Mandelstam & Mcquillan, 1973).

Biosynthesis of Aromatic amino acids

Bacteria and plants could synthesize all three aromatic amino acids (phenylalanine, tyrosine and tryptophan) in a metabolic process known as the shikimate pathway. The shikimate pathway included the production of chorismate (Figure A), which was the common precursor for the three aromatic amino acids. This pathway branched at many points leading to the production of an extensive range of products that include metal chelators, vitamin E and K, folic acid, ubiquinone and plastoquinone (Hawkins, Lamb, Moore, Charles & Roberts, 1993). In higher organisms, phenylalanine and tryptophan are considered nutritionally essential, as they cannot be synthesized de novo. Tyrosine on the other hand, can be synthesized by a hydroxylation reaction of phenylalanine, therefore is considered as a nutritional non-essential amino acid (Haslam, 1974).

Chorismic acid branch point.

The initial stages for aromatic amino acid (phenylalanine, tyrosine and tryptophan) synthesis were the same until chorismic acid. Figure A illustrates detail steps

of the pre-chorismate shikimate pathway as seen in those prokaryotes, eukaryotes and plants studied (Haslam, 1974).

Figure B depicts the post chorismate shikimate pathway. Chorismate mutase (A) catalyzes the conversion of chorismic acid to prephenic acid (Figure B). In *Pseudomonas* species chorismate mutase had an optimal activity at 33^0C and the enzyme synthesis was induced by the presence of tryptophan in the culture medium (Keller, Keller, Klages & Lingens, 1983). At this branch point, in *Brevibacterium flavum*, the velocity of tryptophan synthesis (from chorismic acid) was larger than that of phenylalanine and tyrosine biosynthesis. The metabolic flow of chorismate was diverted from tryptophan to phenylalanine and tyrosine synthesis through the feedback inhibition and repression of anthranilate synthetase (the first tryptophan specific enzyme in the pathway) by tryptophan. Tryptophan was also partially responsible for activation of chorismate mutase (A)(Shiio & Sujimoto, 1981).

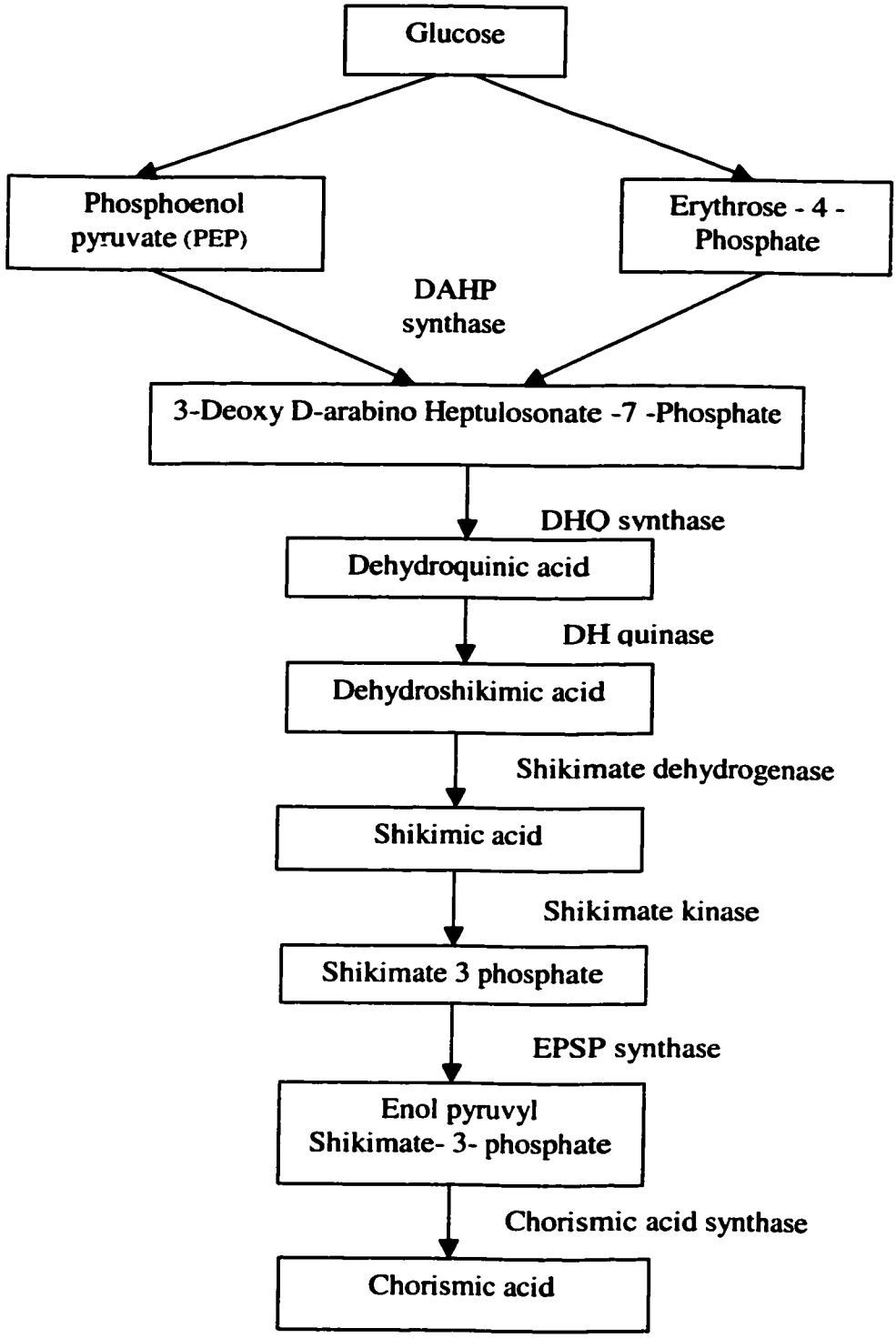


Figure A. Pre-chorismate Shikimate pathway

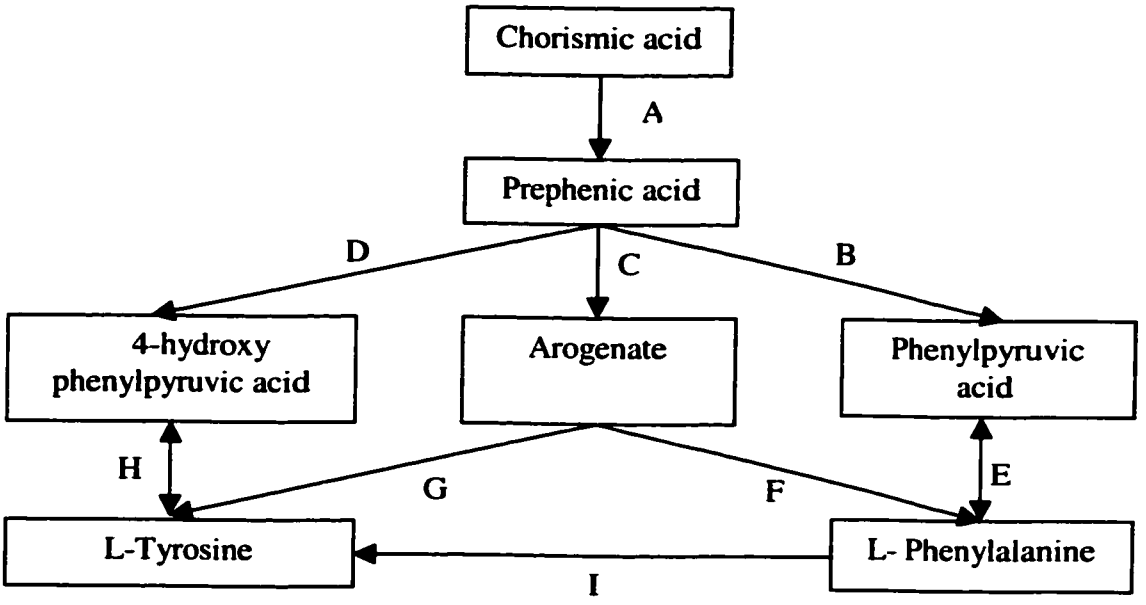


Figure B. Post-chorismic acid shikimate pathway

A-Chorismate Mutase, B- Prephenate dehydratase, C- Prephenate transaminase, D- Prephenate dehydrogenase, E- Phenylalanine transaminase, F- Arogenate dehydratase, G- Arogenate dehydrogenase, H- Tyrosine transaminase, I- Phenylalanine hydroxylase

Prephenic acid branch point.

Prephenate was another important branch point, in phenylalanine/tyrosine biosynthesis. Prephenate dehydratase (B) and prephenate dehydrogenase (D) catalyzed the conversion of prephenate to phenylpyruvic acid (phenylalanine specific precursor) and 4-hydroxyphenylpyruvic acid (tyrosine specific precursor) respectively (Haslam, 1974). In bacteria such as *Pseudomonas* species, prephenate dehydratase (B) had a temperature optimum of 30^0C. Prephenate dehydrogenase (D) accepted both NAD as well as NADP as hydrogen acceptors. The activity of prephenate dehydrogenase (D) was reduced to 50 % of the control in the presence of tyrosine (Keller *et al.*, 1983). Regulation of phenylalanine synthesis occurred at this point, where prephenate dehydratase (B) was inhibited by phenylalanine and activated by tyrosine in *A. methanolica.* Studies with this bacterium indicated that the presence of tyrosine in the assay medium increased the activity of prephenate dehydratase (B) by 300 % of the control (Abou-Zeid, Euverink, Hessels, Jensen & Dijkhuizen, 1995).

The presence of an alternate pathway for tyrosine biosynthesis was described in cyanobacteria (Stenmark, Pierson, Glover & Jensen, 1974), *P. aeruginosa* (Patel, Pierson & Jensen, 1977) and coryneform bacteria (Fazel & Jensen, 1979). A prephenate transaminase (C) was involved at this point, converting prephenate to arogenate, in some species, especially in bacteria. Keller, Keller, Salcher and Lingens (1982) had reported similar results in *Pseudomonas aureofaciens,* suggesting tyrosine may be synthesized via three routes, the 4-hydroxy phenylpyruvate and arogenate pathways as well as

phenylalanine via the phenylpyruvate. Two different kinds of prephenate dehydratases (B), I and II were known in *P. aeruginosa* PAO1 (Calhoun, Pierson, & Jensen, 1973; Patel, Pierson, & Jensen, 1977). In *P. aureofaciens*, prephenate dehydratase I was associated with chorismate mutase, activated by tyrosine and inhibited by phenylalanine. Either product did not influence prephenate dehydratase II (Blumenstock, Salcher & Lingens, 1980). While prephenate dehydratase I did not show any activity with arogenate, prephenate dehydratase II was able to react with both prephenate and arogenate converting them to their respective end products (Patel *et al.*, 1977). There was a 250 fold excess of prephenate dehydratase I activity in a crude extract of *P. aureofaciens* ATCC 15926 compared to arogenate dehydratase. This suggested that phenylpyruvate branch might be the main route of phenylalanine synthesis in *P. aureofaciens* (Keller *et al.*, 1982).

Phenylalanine/ Tyrosine transaminase.

The conversion of phenylpyruvic acid to phenylalanine is a reversible reaction involving the enzyme phenylalanine transaminase (E). Tyrosine transaminase (H) is also a reversible enzyme, that converts 4-hydroxy phenylpyruvic acid to tyrosine (Nyhan, 1967). In *A. methanolica* two isoenzymes (multiple forms in which an enzyme can exist in an organism), were classified with phenylalanine/tyrosine transaminase activity. Phenylalanine/Tyrosine transaminase I normally functioned as a branched chain amino acid amino transferase and had a broad range of substrate specificity. Phenylalanine/Tyrosine transaminase II was the dominant amino transferase for L-

phenylalanine biosynthesis and also catalyzed the first step of tyrosine catabolism (to 4-hydroxyphenylpyruvic acid). Biosynthesis of tyrosine involved the two enzymes prephenate transaminase (C) and arogenate dehydrogenase (G) (Abou-Zeid *et al.*, 1995). In *Pseudomonas* species, arogenate dehydratase (F) synthesis was repressed by tyrosine. The activity of arogenate dehydrogenase (G) was inhibited by tyrosine in the assay medium (Keller *et al.*, 1983).

Phenylalanine hydroxylase.

Phenylalanine hydroxylase (I) was the enzyme responsible for the conversion of phenylalanine to tyrosine. This aryl hydroxylation reaction occurred in the liver of all higher animals and in some plants and bacteria. In most plants this interconversion does not occur; instead phenylalanine and tyrosine were synthesized independently (Haslam, 1974).

According to Bender (1985) three proteins were involved in the hydroxylation of phenylalanine to tyrosine in higher animals. Phenylalanine hydroxylase which consisted of a biopterin dependent mixed function oxidase, an NADPH dependent dihydrobiopterin reductase, and a stimulator protein. The enzyme had two factors, the stable and labile factors. The stable factor is widely distributed in all tissues. The labile factor which was found essentially only in the liver was responsible for the conversion of phenylalanine to tyrosine. The phenylalanine hydroxylase found in the liver cells of mammals was found in the soluble fraction. It required oxygen and NADPH and was specific for phenylalanine (Nyhan, 1984).

Conversion of phenylalanine to tyrosine and hence, phenylalanine catabolism were regulated by changes in the activity of phenylalanine hydroxylase, which was regulated by its phosphorylation state. Phosphorylation of a seryl residue of the enzyme activates the phenylalanine hydroxylase by increasing its specific activity. Phenylalanine also activates the enzyme. The potency of phenylalanine as an activator of the enzyme is greater for the phosphorylated form of the enzyme (Doskeland, Martinez, Knappskog, & Flatmark, 1996; Kaufmann, 1993).

A phenylalanine hydroxylase gene has been identified in the nematode, *C. elegans*. Loer, Davidson and Mckerrow (1999) have hypothesized that the gene K08F8.4 could be assigned the role of encoding for phenylalanine hydroxylase.

Phenylketonuria is a genetic disorder of phenylalanine metabolism, which in homozygous individuals results in an inability to synthesize phenylalanine hydroxylase in an active form. This results in hyperphenylalaninemia, and phenylpyruvic aciduria. The excess phenylalanine is catabolized to phenylpyruvic acid, phenylacetic acid and phenyllactic acid. Phenylacetic acid in sweat and urine produces a characteristic musty odor. Other biochemical and clinical manifestations of the disorder include excessive production and excretion of 4-hydroxy phenylacetic acid, alteration of tryptophan metabolism, changes in pigmentation and mental retardation (Auerbach, DiGeorge & Carpenter, 1984).

CHAPTER 2 JOURNAL ARTICLE

PHENYLALANINE REQUIREMENT AND BLOCKAGE IN ITS BIOSYNTHESIS IN *C. ELEGANS*

BY

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1 Summary

Phenylalanine requirement, optimal phenylalanine-tyrosine combination and the sparing effect between the two amino acids was determined in the free-living nematode, *C. elegans*. The optimal requirement of phenylalanine ranged between 1.1-4.2 mg/ml. The optimal phenylalanine - tyrosine combination was established as 0.53: 0.27 mg/ml. Phenylalanine completely spared tyrosine requirement at the optimal range established. At 0.27 mg/ml tyrosine spared as high as 75 % of phenylalanine requirement. The site of blockage in the biosynthetic pathway of phenylalanine was determined by supplementing precursors of phenylalanine in the medium. 85 - 92 % blockage was noticed with phenylpyruvic acid. Major blockage (100 %) occurred with prephenic acid. Conclusive evidence of additional blockage with shikimic acid and chorismic acid was not indicated.

Keywords: Aromatic amino acids, tyrosine, sparing effect, nematodes

Caenorhabditis elegans (C. elegans) recently achieved the status of being the only animal model with its entire genome sequenced (The *C. elegans* Consortium, 1998). Up to 83 % similarity to human genes has been illustrated by comparative genome identity studies, (Lai *et al.*, 2000). However, *Caenorhabditis briggsae* (*C. briggsae*) was isolated as early as 1944 and was used as an animal research model by Dougherty *et al.* (1959). *C. briggsae* was demonstrated to be a closely related nematode differing from *C. elegans* only in genetic composition and the ability to interbreed (Friedman *et al.*, 1977; Nicholas, 1984). The *C. briggsae* Maintenance Medium was established by Buecher *et al* (1966). The requirement of sterol (Hieb & Rothstein, 1968) heme (Hieb, Stockstad & Rothstein, 1970) and acetate (Lu *et al*, 1978) in the nematode was later established. The final completely defined chemical medium (*C. elegans* Maintenance Medium, CeMM) was developed in 1993, which had glucose as an energy source (Lu & Goetsch, 1993).

Most nutritional requirements of *C. elegans* have been shown to be similar to that of higher organisms, including essential amino acids (Vanfleteren, 1973). The requirement for vitamins (Lu *et al.*, 1974; Sun *et al.*, 1986; Augustin *et al.*, 1994; Li *et al.*, 1995; Pothana *et al.*, 1997) and minerals (Lu *et al.*, 1983) have been established. The CeMM contains essential and non-essential amino acid concentrations modeled on the composition of *Escherichia coli*, which was used as the primary food source for this nematode in the monoxenic culture (Edgar & Wood, 1977). The requirements for branched chain amino acids have been recently established (Perelman & Lu, 2000).

Phenylalanine has been demonstrated to be an essential amino acid for most nematodes (Vanfleteren, 1973; Hata, 1994; Jackson, 1973; Brockelman & Jackson, 1978;

Myers & Balasubramaniam, 1973). The first step in phenylalanine catabolism was its hydroxylation to tyrosine. Phenylalanine hydroxylase was the enzyme responsible for the conversion of phenylalanine to tyrosine (Nyhan, 1984). This aryl hydroxylation occurred in the liver of all higher animals and in some plants and bacteria (Haslam, 1974). Since the gene encoding phenylalanine hydroxylase, was recently reported to be present in the hypodermis of *C. elegans* (Loer *et al.*, 1999), it is possible that *C. elegans* would be able to synthesize tyrosine from phenylalanine. Studies about the biosynthetic pathway of phenylalanine and tyrosine have been extensive with microorganisms such as bacteria (Mosin *et al.*,1998), yeast (Fiske & Kane,1984), and fungi (Giles *et al.*, 1985).

The objectives of this study were to determine the quantitative requirement of phenylalanine in a tyrosine free *C. elegans* maintenance medium (CeMM) and to establish an optimal phenylalanine-tyrosine concentration combination. Simultaneously the sparing effect between the two amino acids was examined. The site of blockage in the biosynthetic pathway of phenylalanine in the nematode was also investigated, using the precursors of phenylalanine such as shikimic acid, chorismic acid, prephenic acid and phenylpyruvic acid.

2 Materials and Methods

The study was conducted in two phases. In the first phase, optimal requirement of phenylalanine and the optimal phenylalanine - tyrosine concentration combination was established. The sparing effect between the two amino acids was also determined in this phase. In the second phase, the site of blockage in the biosynthesis of phenylalanine was investigated. This was investigated by substituting shikimic acid, chorismic acid, prephenic acid and phenylpyruvic acid at equimolar concentrations of the optimal phenylalanine concentration that was established in the first phase.

2.1 Stock Medium and Nematode Culture

The stock medium (HS - YE- HLE) used for cultivating the nematodes consisted of 4 % Hi-Soy (Quest International, Chicago, IL, USA), 1 % yeast extract (Sigma, Saint Louis, MO, USA) (Tomlinson & Rothstein, 1962) and 10 % heated liver extract (Sayre *et al.*, 1963). Cultures were incubated at 20 °C on a tissue culture rotator for approximately two weeks. Nematodes were harvested by centrifugation, washing and re-suspending in distilled water for use as innoculum in experimental media (Lu *et al.*, 1983). Approximately 2500 nematodes in 0.1 ml distilled water were inoculated in each 5-ml experimental medium resulting in an initial population of 500 nematodes/ml. Each concentration level was done in quadruplicates and each experiment was repeated at least twice to test the validity of the results.

2.2 Requirement of Phenylalanine

C. elegans Maintenance Medium (CeMM) (Lu & Goetsch, 1993) with both phenylalanine and tyrosine deleted (- Phe - Tyr) was used as the basal medium for nematode culture. Glucose was used as the energy source in this basal medium. The nutritional status of the nematodes was assessed using the nematode's population growth as an index.

a. The optimal requirement of phenylalanine was determined by supplementing the basal medium (CeMM - Phe - Tyr) with seven concentrations of phenylalanine at 0, 0.26, 0.53, 1.1(standard CeMM level), 2.1, 4.2 and 8.4 mg/ml of the medium.

b. The optimal phenylalanine - tyrosine concentration combination was determined by utilizing a 4 x 4 factorial design. Four concentrations of phenylalanine - 2.1 (optimal phe concentration determined from experiment (a) above), 1.1 (1/2 optimal), 0.53 (1/4 optimal) and 0.0 mg/ml and four concentrations of tyrosine at 0.54 (2x CeMM level), 0.27 (CeMM level), 0.14 (1/2 CeMM level) and 0.0 mg/ml were combined. These concentration combinations were supplemented in the CeMM (- Phe - Tyr) to give 16 different media. This experiment was designed to investigate the sparing effect between phenylalanine and tyrosine as well.

c. The presence of any complete sparing effect of tyrosine on phenylalanine requirement was further investigated by supplementing tyrosine in the CeMM (-Phe - Tyr) at 1.2 mg/ml. This level was based on the total amount of phenylalanine concentration (0.80 mg/ml) plus the tyrosine concentration (0.27 mg/ml) in the CeMM.

2.3 Blockage in Biosynthetic Pathway

The basal medium (- Phe -Tyr) was supplemented with phenylalanine precursors - shikimic acid (2.2 mg/ml), chorismic acid (2.8 mg/ml), prephenic acid (2.8 mg/ml) and phenylpyruvic acid (2.1 mg/ml), at molar concentrations of the optimum level of phenylalanine established previously in this study.

CeMM with all its original components was used as a control for comparison of the growth in nematode population, in all the experiments. In each experiment, nematode population growth was assessed by counting all the live nematodes at 7, 14 and 21 days. The 21st day population growth was used as the index for treatment effect.

2.4 Statistical Analysis

Multiple comparisons with paired t-tests were used to test the significance between concentrations. Bonferroni method was adopted to adjust the p-value to 0.05. Standard deviation and percentages were used for estimating the sparing effect and blockage in biosynthesis. Data analysis tool in Microsoft Excel was used in all of the above analyses.

3 Results

3.1 Requirement of Phenylalanine

The maximum population growth (15 x 10^4 nematodes/ml) in the nematodes was established at the concentration of 2.1 mg/ml of phenylalanine in a tyrosine free medium (Figure 1). With decrease in concentration to 1.1 mg/ml (CeMM level) a 23 % decline in population growth (11 x 10^4 nematodes/ml) was noticed. When the concentration of phenylalanine was increased above 2.1 mg/ml to 4.2 mg/ml of the medium there was a 36 % decline in growth. However, no significant difference ($p < 0.05$) was seen between the concentrations 1.1, 2.1 and 4.2 mg/ml. Hence, 1.1 - 4.2 mg/ml of phenylalanine in the medium was considered as the optimal concentration range. Further, since 2.1 mg/ml of phenylalanine yielded maximum population growth in both trials, it was considered as the optimal concentration for subsequent experiments in this study. At other higher (8.4 mg/ml) and lower concentrations (0.53, 0.27 & 0.0 mg/ml) of phenylalanine a significantly lowered ($p < 0.05$) growth response was seen. These concentrations were then considered toxic and deficient respectively. Although no population growth was attained at the lowest two levels, minimal growth (3.2 x 10^4 nematodes/ml) was noticed at 0.53 mg/ml of phenylalanine.

Table 1 indicates the population growth of the nematodes with various phenylalanine-tyrosine concentration combinations. The maximum growth in population was attained when the phenylalanine and tyrosine combination was 2.1: 0.0 mg/ml (13 x 10^4 nematodes/ml). There was no significant difference ($p < 0.05$) between seven

concentration combinations of phenylalanine and tyrosine. They were 2.1:0.0, 2.1:0.14, 1.1:0.27, 1.1:0.14, 1.1:0.0, 0.53:0.54 and 0.53:0.27 mg/ml of phenylalanine and tyrosine concentration combinations. Among these combinations, 0.53:0.27 mg/ml of phenylalanine and tyrosine, could be considered the most efficient, since optimal population growth was attained with minimal amount of the two nutrients. At the highest concentration of tyrosine (0.54 mg/ml) used in the experiment, the maximum population growth (12 x 10^4 nematodes/ml) was obtained at a phenylalanine concentration of only 0.53 mg/ml. This indicated that increase in tyrosine concentrations resulted in a decrease in phenylalanine requirement in the nematodes. At concentration combinations where both phenylalanine and tyrosine were the highest (2.1:0.54 mg/ml) lower population growth was attained, which indicated toxicity effects. Similarly at lowest concentration combinations (0.53:0.14 mg/ml) of phenylalanine and tyrosine a reduced population growth was noticed indicating deficiency effects.

At the optimal concentration combination of phenylalanine and tyrosine established earlier (0.53: 0.27 mg/ml), tyrosine spared 75 % of phenylalanine requirement (at 2.1 mg/ml) in *C. elegans*. In other words at 0.27 mg/ml of tyrosine, only 0.53 mg/ml of phenylalanine was required as opposed to 2.1 mg/ml when tyrosine was completely eliminated from the medium. A sparing effect of 75 % was noticed at a higher level (0.54 mg/ml) of tyrosine as well. In order to investigate any further sparing effect of high concentrations of tyrosine, phenylalanine was completely eliminated from the medium and tyrosine concentration was increased to 1.2 mg/ml. From Table 2 it is evident that

tyrosine could not support growth in the absence of phenylalanine, since there was absolutely no population growth of the nematodes.

3.2 Blockage in Biosynthetic Pathway

Table 3 presents the population growth of *C. elegans* with various precursors of phenylalanine. Insufficient growth response in *C. elegans* was noticed with all the four precursors tested. There was less than one percent growth, with shikimic, chorismic and prephenic acids when compared with CeMM (100 %). However an 8.7 % growth (1.6 x 10^4 nematodes/ml) was noticed with phenylpyruvic acid (the immediate precursor of phenylalanine). In another experiment (Table 4), the concentration of phenylpyruvic acid was increased from 2.1 mg/ml to 4.2 and 8.4 mg/ml. Although a 15 % growth (1.7 x 10^4 nematodes/ml) was noticed with 2.1 mg/ml of phenylpyruvic acid (Table 4), corresponding increase in population growth was not evident with increase in phenylpyruvic acid concentrations. Growth was suppressed at 4.2 mg/ml and 8.4 mg/ml of phenylpyruvic acid.

4 Discussion

The maximum population growth in *C. elegans* was demonstrated at 2.1 mg/ml of phenylalanine in a tyrosine-free medium. However, the optimal requirement of phenylalanine in a tyrosine-free medium ranged between 1.1 - 4.2 mg/ml. At 2.1 mg/ml of phenylalanine, tyrosine can be completely eliminated from the medium. The combined phenylalanine-tyrosine levels in their respective media for other nematodes such as *Neoaplectana glaseri* (0.08 mg/ml) (Jackson, 1973), *Rhabditis maupausi* (0.08

mg/ml)(Brockelman & Jackson, 1978), and *Aphelenchoides rutgersi* (0.15 mg/ml) (Myers & Balasubramaniam, 1973) were lower than the requirement established in this study (2.1mg/ml). A tyrosine level of 0.08 mg/ml was also present in the medium used for growth of *N. glaseri*.

An optimal concentration combination of phenylalanine and tyrosine at 0.53: 0.27 mg/ml was established in this study. This is almost similar to the ratio of 0.62: 0.27 mg/ml described by Vanfleteren (1973) and the ratio present in the CeMM (0.80:0.27 mg/ml) for optimal reproduction in *C. briggsae*. As discussed earlier, at 2.1 mg/ml of phenylalanine, tyrosine could be completely eliminated. This could be adopted in CeMM to overcome the problem of low solubility of tyrosine (Lu & Goetsch, 1983). Further, the 0.53: 0.27 mg/ml of phenylalanine - tyrosine could be considered as the most efficient combination as minimal amount of the two nutrients (on a weight basis) was utilized to produce optimal growth.

Since phenylalanine could completely replace tyrosine requirement of the nematode, it is possible that the enzyme responsible for the hydroxylation of phenylalanine to tyrosine, phenylalanine hydroxylase, was present in the nematode. According to Bender (1985) three proteins were involved in the hydroxylation of phenylalanine to tyrosine: 1) phenylalanine hydroxylase which is a biopterin dependent mixed function oxidase, 2) dihydrobiopterin reductase, which is NADPH dependent, and 3) a stimulator protein. It can therefore be presumed that all the three proteins were actively present in *C. elegans*. The results concurred with the findings of Loer *et al*

(1999), who had identified a gene encoding phenylalanine hydroxylase in the hypodermis of *C. elegans*. On the other hand, there was no growth in the nematode when tyrosine was used to completely substitute for phenylalanine. This agreed with the previous study (Vanfleteren, 1973) that showed that phenylalanine was an essential amino acid for *C. elegans*, whereas tyrosine was not. Corresponding results from studies with other nematodes such as *Angiostrongylus costaricencis* (Hata, 1994), *Neoaplectana glaseri* (Jackson, 1973), *Rhabditis maupasi* (Brockelman & Jackson, 1978) *Aphelenchoides rutgersi* (Myers & Balasubramaniam, 1973), and *Caenorabditis briggsae* (Vanfleteren, 1973) have demonstrated phenylalanine to be essential. Tyrosine has not been demonstrated to be essential in any of these nematodes except *Neoaplectana glaseri*, in which delayed reproduction and reduced population growth was noticed in a tyrosine deficient medium. Tyrosine was demonstrated to be partially essential in this nematode (Jackson, 1973).

Since optimal growth was established in a tyrosine free medium at 2.1 mg/ml of phenylalanine, it can be presumed that phenylalanine was used as a tyrosine precursor, when the latter was absent or deficient in the medium. Phenylalanine therefore was demonstrated to spare tyrosine requirement completely in *C. elegans*. Tyrosine at concentrations ranging from, 0.27-0.54 mg/ml could spare upto 75% of phenylalanine requirement. To elucidate, at tyrosine concentrations of 0.27 mg/ml in the medium, phenylalanine requirement was reduced from 2.1 mg/ml (in a tyrosine free medium) to 0.53 mg/ml. Even though phenylalanine was required for growth, its requirement was substantially reduced. This could also be interpreted as even though tyrosine is a non -

essential amino acid for the nematode, it could spare phenylalanine in a phenylalanine deficient state. In a phenylalanine deficient state, therefore tyrosine could be considered as a conditionally essential amino acid for this nematode. This sparing effect of tyrosine was reported in higher organisms such as kittens (Williams *et al.*, 1987), chicks (Sasse & Baker, 1972), and lactating sows (Lellis & Speer, 1987). No such studies have been done so far with nematodes.

From our study of the biosynthetic pathway of phenylalanine in *C. elegans*, it was demonstrated that the major blockage in the pathway could be at the prephenic acid point. The conversion of phenylpyruvic acid to phenylalanine was reportedly a reversible reaction involving the enzyme phenylalanine transaminase (Bender, 1985). In this study there was an 8 - 15% growth at 2.1 mg/ml of phenylpyruvic acid, which suggested that limited amount of phenylalanine might have been synthesized. When the concentrations were increased there was no growth in nematode population. This might be due to the toxicity associated with the acidemia produced with excess phenylpyruvic acid. Hence, even if phenylpyruvic acid could convert to phenylalanine, higher levels of phenylpyruvic acid might have produced an acidic pH, which would not be conducive to *C. elegans* growth. Moreover, the enzyme phenylalanine transaminase, which is responsible for the transamination of phenylpyruvic acid to phenylalanine, has optimal activity at 55^0C and a pH of 8 in the bacterium, *Amycolatopsis methanolica* (Abou-Zeid *et al.*, 1995). Whereas, *C. elegans* is cultured at 20^0C and the CeMM is maintained at a pH of 5.9 (Lu & Goetsch, 1993). The enzyme conditions that were established in *A. methanolica* might not be reflected in *C. elegans*. However, further minute increase in

phenylpyruvic acid concentrations and manipulation of culture conditions would reveal additional information regarding this aspect.

Significant growth was not seen at the prephenic acid, chorismic acid and shikimic acid points. Shikimic acid is the first precursor in the biosynthetic pathway among the precursors investigated (Figure 2). There are two intermediates between shikimic acid and chorismic acid, shikimate-3-phosphate and enol-pyruvyl shikimate-3-phosphate (Hawkins *et al.*, 1993). These were not tested, as they were not available commercially. Chorismic acid is the branch point at which another aromatic amino acid, tryptophan synthesis diverges from phenylalanine/tyrosine synthesis. The enzyme, chorismate mutase directs phenylalanine/tyrosine synthesis. It is at this point that folic acid and ubiquinone synthesis also diverged (Haslam, 1974). Prephenic acid is the branch point in the shikimate pathway, where phenylalanine and tyrosine synthesis diverge in most prokaryotes and eukaryotes studied. While prephenate dehydratase is the phenylalanine specific enzyme, prephenate dehydrogenase is the tyrosine specific enzyme (Haslam, 1974). If there had been a minimal growth with prephenic acid, as that noticed with phenylpyruvic acid, we could assume that there was no blockage between prephenic acid and phenylpyruvic acid. Similar assumptions could be made with the other precursors, provided a minimal growth was attained. The lack of growth on the other hand does not indicate complete blockage in the other sites investigated as these precursors might have been substituted in toxic concentrations. Therefore, based on the findings we concluded that the major blockage in the biosynthetic pathway occurred between phenylpyruvic acid and prephenic acid.

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7 Tables

Table 1. **Population growth of *C. elegans* in various Phenylalanine-Tyrosine concentration combinations**

		Population Growth (x 10^4 nematodes/ml)			
		Tyrosine concentrations (mg/ml)			
		0.54	**0.27 (CeMM)**	**0.14**	**0.0**
Phenylalanine concentrations (mg/ml)	**2.1**	7.9 ± 0.56 (b)	9.9 ± 0.58 (b)	11 ± 2.0 (a)	13 ± 0.58 (a)
	1.1	8.8 ± 0.20 (b)	12 ± 1.0 (a)	10 ± 0.60 (a)	11 ± 1.0 (a)
	0.53	12 ± 0.90 (a)	12 ± 1.3 (a)	6.3 ± 0.88 (b)	7.1 ± 0.45 (b)
	0.0	0.018 ± 0.0043 (c)	0.018 ± 0.0089 (c)	0.017 ± 0.0013 (c)	0.0071 ± 0.0058 (c)

a, b,c = significantly different ($p < 0.05$).

Each tube was inoculated with 500-nematodes/ml containing 5-ml medium/tube. Each trial value is the mean population of four tubes.

Table 2. Population growth of *C. elegans* in a phenylalanine-free medium

Concentrations	Population Growth (x 10^4 nematodes/ml)
Tyrosine (1.2 mg/ml) *	0.0025 ± 0.002
CeMM (0.80 mg/ml Phe, 0.27 mg/ml Tyr)	19 ± 1.2

* The concentration was derived by the supplementing additional tyrosine to the original tyrosine concentration (0.27 mg/ml) to replace phenylalanine (0.80 mg/ml) in CeMM. Each tube was inoculated with 500-nematodes/ml containing 5-ml medium/tube. Each trial value is the mean population of four tubes.

Table 3. Population growth of *C. elegans* in precursors of phenylalanine

Precursors		Population Growth (x 10^4 nematodes/ml)	% of control
Name	Concentration (mg/ml)		
Shikimic acid	2.2	0.04 ± 0.0052	< 1% growth
Chorismic acid	2.8	0.11 ± 0.0088	< 1% growth
Prephenic acid	2.8	0.06 ± 0.0066	< 1% growth
Phenylpyruvic acid	2.1	1.6 ± 0.16	8.7%
Phenylalanine	2.1	15 ± 2.8	79 %
CeMM (control)	0.80 (phe); 0.27 (tyr)	19 ± 1.2	100 %

Each tube was inoculated with 500-nematodes/ml containing 5-ml medium/tube. Each value of the population growth represents the mean of the four trials conducted per precursor.

* The precursors were supplemented at equimolar concentrations of the optimal level of phenylalanine established earlier (2.1 mg/ml Phenylalanine) in a basal media without phenylalanine or tyrosine (CeMM minus Phenylalanine &Tyrosine).

Table 4. **Population growth of *C. elegans* in various concentrations of phenylpyruvic acid**

Phenylpyruvic acid (mg/ml)	Population growth (10^4 nematodes/ml)	Standard deviation (10^4 nematodes/ml)	% of control
2.1	1.7	± 0.11	14 %
4.2	44	± 0.037	0 %
8.4	0	± 0	0 %
Phenylalanine - 2.1 mg/ml	13	± 0.58	108 %
CeMM - 0.80 (phe); 0.27 (tyr) mg/ml	12	± 1.2	100 %

Each tube was inoculated with 500-nematodes/ml containing 5-ml medium/tube. Each value of the population growth represents the mean of the four trials conducted per concentration.

2.1 mg/ml was the optimal concentration of phenylalanine established previously (Table 1).

8 Figures

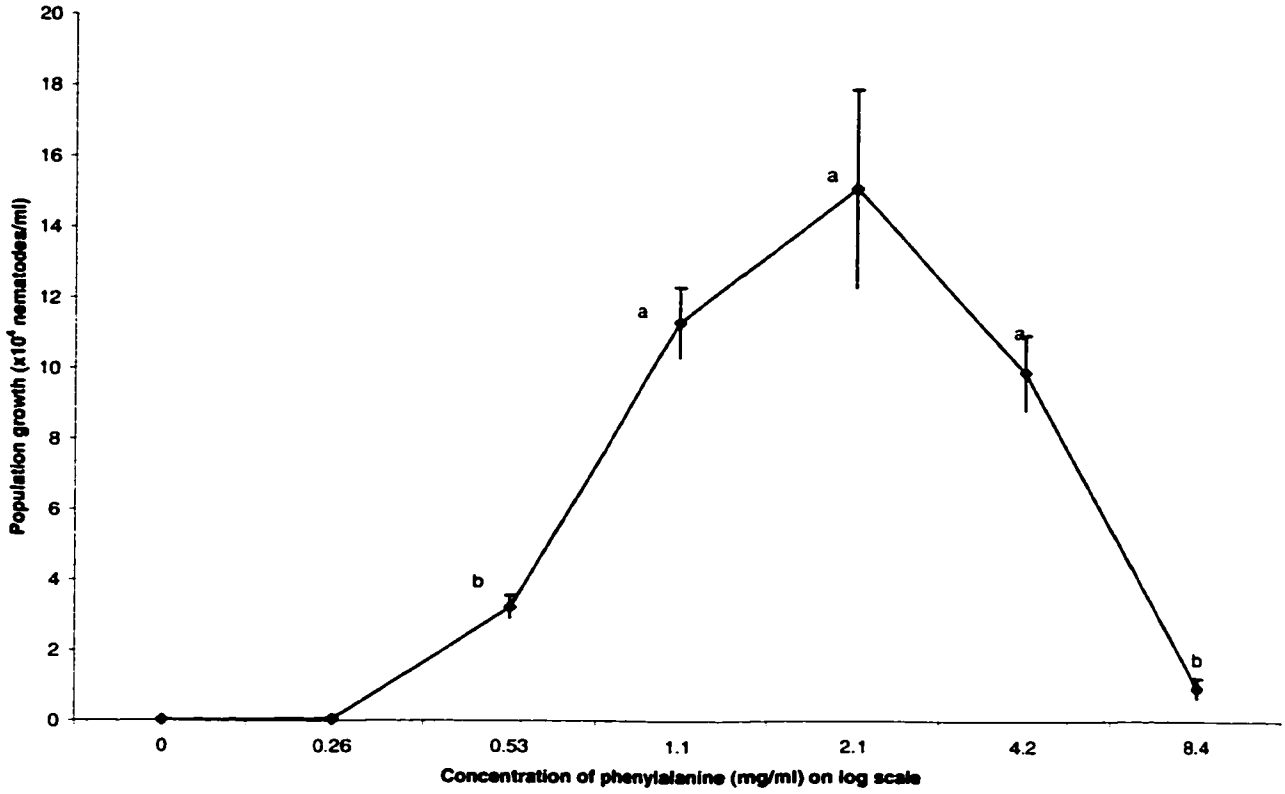

Figure 1. Population growths of *Caenorhabditis elegans* in various concentrations of phenylalanine in a tyrosine free CeMM. Five hundred nematodes per ml of medium were inoculated in each tube and the population growth was determined 21 days after inoculation. Bars represent ± standard deviation of the mean of four replicates; a, b = significantly different.

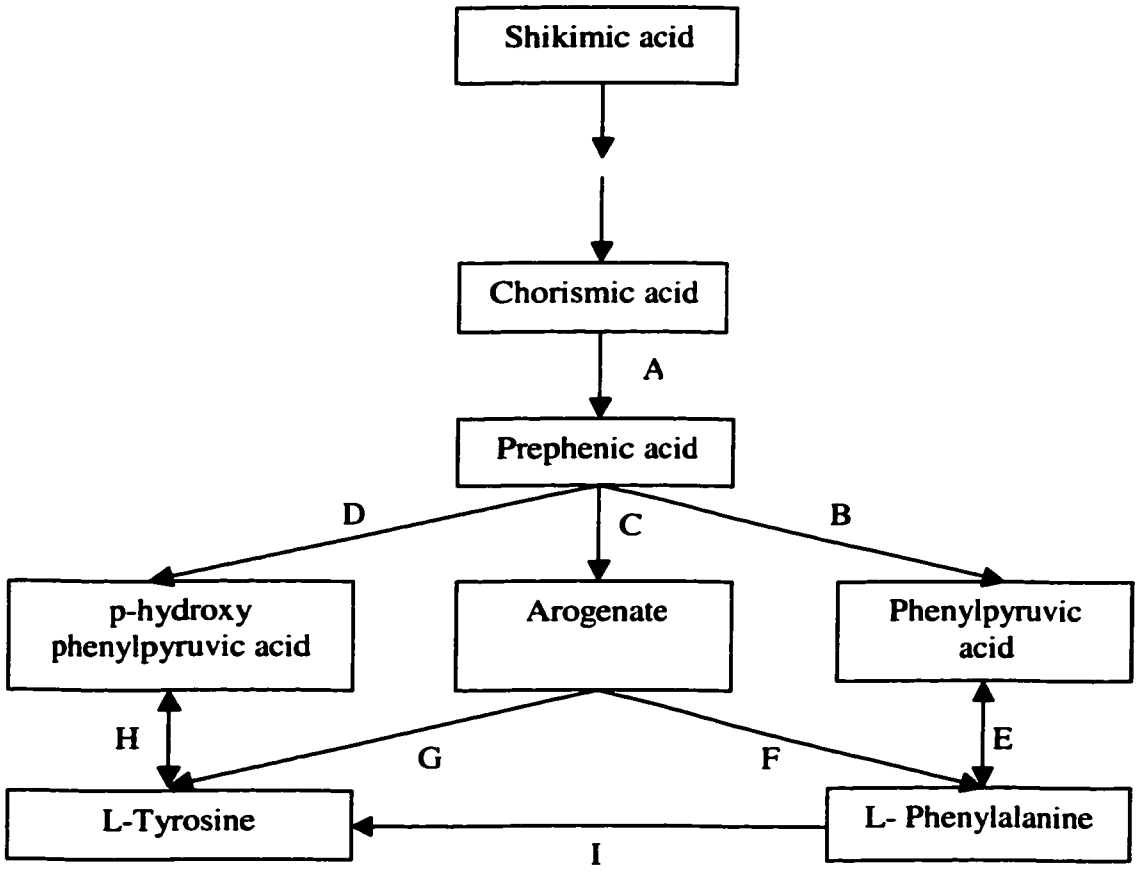

Figure 2. Biosynthesis of Phenylalanine/Tyrosine from shikimic acid

A-Chorismate Mutase, B- Prephenate dehydratase, C- Prephenate transaminase, D- Prephenate dehydrogenase, E- Phenylalanine transaminase, F- Arogenate dehydratase, G- Arogenate dehydrogenase, H- Tyrosine transaminase, I- Phenylalanine hydroxylase

Chapter 3

Summary and Recommendations

Summary

Phenylalanine requirement, optimal phenylalanine-tyrosine combination and the sparing effect between the two amino acids was determined in the free-living nematode, *C. elegans*. The optimal requirement of phenylalanine ranged between 1.1-4.2 mg/ml. The optimal concentration combination of phenylalanine and tyrosine was established as 0.53: 0.27 mg/ml. Phenylalanine completely spared tyrosine requirement at the optimal range established. At 0.27 mg/ml tyrosine spared as high as 75 % of phenylalanine requirement. The site of blockage in the biosynthetic pathway of phenylalanine was determined by supplementing precursors of phenylalanine in the medium. An 8-15 % growth in population was noticed with phenylpyruvic acid. No growth was noticed with the other precursors. These results indicated that around 85-92 % blockage occurred between phenylpyruvic acid and phenylalanine. However, additional blockages also occurred before phenylpyruvic acid. Therefore, we concluded that the major blockage in the biosynthetic pathway occurred between phenylpyruvic acid and prephenic acid.

Recommendations for Further Research

The following suggestions are made for further research using *C. elegans* as a model for studying amino acid metabolism.

1. The presence of slight growth with phenylpyruvic acid could be further investigated with lesser increments than the ones used in this study (i.e 1.4, 2.1, 2.8, 3.5 and 4.2 mg/ml of phenylpyruvic acid).

2. An alternate pathway for the biosynthesis of tyrosine has been demonstrated in bacteria. The existence of such a pathway in *C. elegans* could be explored.

3. *Caenorhabditis elegans* could be used as a model to investigate the biosynthesis of the other essential amino acids that have not yet been researched.

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Appendix I

CAENORHABDITIS ELEGANS MAINTENANCE MEDIUM (Lu & Goetsch, 1993)

I	**Vitamins & Growth Factors**	**Molecular Weight**	**Grams***
	N- Acetylglucosamine	222.2	0.01500
	Cyanocobalamine	1355.3	0.00375
	Niacinamide	122.1	0.00750
	Pantethine	554.7	0.00375
	Pantothenate (Ca)	238.3	0.00750
	Pyridoxamine.2HCl	241.1	0.00375
	Pyridoxine.HCl	205.6	0.00750
	Pyridoxal.PO_4	247.1	0.00375
	Riboflavin-5'- PO_4 (Na).$2H_20$	514.4	0.00750
	Thiamin.HCl	337.3	0.00750
	Biotin	244.3	0.00375
	Niacin	123.1	0.00750
	Pterolylglutamic Acid	441.4	0.00750
	DL-Thioctic Acid	206.3	0.00375
	p- Aminobenzoic Acid	137.1	0.00750
II.	**Salts**		
	$CaCl_2$. $2H_20$	147.0	0.2205
	$CuCl_2$. H_20	170.5	0.0065
	$MnCl_2$.4H2O	197.9	0.0222
	$ZnCl_2$	136.3	0.0102
	KH_2PO_4	136.1	1.2255
	K_2Citrate.H_2O	324.4	0.4860
	$Fe(NH4)_2 (SO_4)_2.6H_2O$	392.2	0.0588
	$Mg(OH)_2$	58.3	0.1740
	Citric Acid.H_2O	210.1	0.6303
III.	**Amino Acids**		
	A. Essential Amino Acids		
	L - Arginine	174.2	0.9750
	L - Histidine	155.2	0.2830
	L - Lysine.HCl	182.6	1.2830
	L - Tryptophan	204.2	0.1840
	L - Methionine	149.2	0.3890
	L - Threonine	119.1	0.7170
	L - Leucine	131.2	1.4390

Appendix I (continued)

	A. Essential Amino Acids (continued)	Molecular Weight	Grams*
	L - Isoleucine	131.2	0.8610
	L - Valine	117.1	1.0200
	L - Phenylalanine	165.2	0.6230
IV.	**B. Non - Essential Amino Acids**		
	L- Phenylalanine	165.2	0.1800
	L- Tyrosine	181.2	0.2720
	L - Alanine	89.1	1.3950
	L - Aspartic Acid	133.1	1.6200
	L - Cysteine.HCl.H_2O	175.6	0.0280
	L - Glutamate (Na). H_2O	187.1	0.5500
	L - Glutamine	146.2	1.4630
	Glycine	75.1	0.7220
	L - Proline	115.1	0.6530
	L - Serine	105.1	0.7880
IV.	**Nucleic Acid Substituents**		
	Adenosine - 3' - (2') - Phosphoric Acid.H_2O	365.2	0.3652
	Cytidine - 3' - (2') - Phosphoric Acid	323.2	0.3232
	Guanosine - 3' - (2') - PO_4 $(Na)_2.H_2O$	425.2	0.3632
	Uridine - 3' - (2') - Phosphoric Acid	324.2	0.3242
	Thymine	126.1	0.1261
V.	**Other Growth Factors**		
	Glutathione, reduced	307.3	0.2040
	Choline H_2 Citrate	295.3	0.0885
	Myo- Inositol	180.2	0.0645
	Cytochrome c	12384.0	0.0500
	β- Sitosterol	414.7	0.0500
VI.	**Energy Source**		
	D - Glucose	180.2	32.5000
	Or K Acetate	98.1	5.0000
VII.	**Solvents**		
	KOH	56.1	**
	Triethanolamine (TEA)	149.2	0.0325
	Tween 80	1308.0	1.2500

* gm/500 ml (2X)

** needed for adjustment of pH to 5.9 +- 0.1